

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF
NICOTIANA BENTHAMIANA WITH *DEHALOGENASE* GENE
RESISTANT TO MONOCHLOROACETIC ACID

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UNIVERSITI TEKNOLOGI MALAYSIA

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
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This thesis is dedicated with love and gratitude

To

My Beloved Mother & My Father

(Allahyarhamah Azizah binti Aziz & Mohamed bin Sayuti)

Who taught me the first word to speak,
the first alphabet to write, the first step to take and have raised me to be
the person I am today.

To

My lovely husband:

Khairul Amin bin Othman

My lovely children:

Elisya Ainul Madihah binti Khairul Amin

Umar al Fetih bin Kahirul Amin

Emeer al Faiq bin Khairul Amin

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ABSTRACT

Weeds give adverse effects to crops because of the competition to get nutrients, light and moisture. Many farmers used broad-spectrum herbicide such as monochloroacetic acid (MCA) which is effective at killing a wide range of weeds. Unfortunately, broad-spectrum of herbicide can also kill valuable crops and cause significant losses in agricultural productivity. One of the solutions to this problem is by developing herbicide resistant plant using *dehalogenase D* (*dehD*) gene isolated from *Rhizobium* sp. RC1. A *dehD* gene encoding dehalogenase enzyme that has the capability to degrade monochloroacetic acid (MCA) was isolated and characterized from *Rhizobium* sp. RC1. *dehD* gene was used as herbicide resistance gene and selectable marker gene in *Nicotiana benthamiana* plant transformation. The 798 bp *dehD* gene was inserted into pCAMBIA 1305.2 under the control of the Cauliflower Mosaic Virus 35S (CaMV35S) promoter and designated as pCAMdehD, with a total size of 10,592 bp. A few parameters of *Agrobacterium tumefaciens*-mediated transformation were optimized including hygromycin concentration (40 µg/mL of hygromycin), and the MCA toxicity level to *N. benthamiana* at tissue culture (60 µg/L of MCA) and whole plant stage (2.0 g/L of MCA). pCAMdehD was introduced into *N. benthamiana* via *Agrobacterium* mediated transformation method. Based on the screening of the transformants on MS media containing 60 µg/L MCA, the results showed that *N. benthamiana* was successfully transformed with *dehalogenase D* gene with 50 % of transformation efficiency. The integration and expression of *dehD* gene in *N. benthamiana* were confirmed by PCR, Southern Blotting and reverse transcription PCR. Analysis of leaf-painting assay revealed that transgenic *N. benthamiana* (T₁) was resistant to 4.0 g/L MCA compared to 2.0 g/L for non-transformed plants control. The Chi Square analyses of five transgenic plants (T₁), suggested that the *dehD* gene was segregated according to Mendelian 3:1 ratio. These findings showed that transgenic *N. benthamiana* plant resistant to MCA herbicide was successfully produced.

ABSTRAK

Rumpai memberikan kesan buruk kepada tanaman kerana persaingan untuk mendapatkan nutrien, cahaya dan kelembapan. Ramai petani menggunakan herbisid berspektrum luas seperti asid monokloroasetik (MCA) yang efektif membunuh pelbagai jenis rumpai. Walau bagaimanapun, herbisid berspektrum luas juga boleh membunuh tanaman yang berfaedah dan menyebabkan kerugian dalam hasil pertanian. Salah satu penyelesaian kepada masalah ini ialah menghasilkan tumbuhan yang rintang terhadap herbisid menggunakan *dehalogenase D* (*dehD*) gen daripada *Rhizobium sp.* RC1. Gen *dehD* mengkodkan enzim dehalogenase yang berupaya mendegradasi asid monokloroasetik (MCA) telah dipencilkan dan dicirikan daripada *Rhizobium sp.* RC1. Di dalam kajian ini, gen *dehD* digunakan sebagai gen rintang herbisid dan gen penanda pemilihan dalam transformasi pokok *Nicotiana benthamiana*. Gen *dehD* bersaiz 798bp telah dimasukkan ke dalam pCAMBIA 1305.2 di bawah kawalan promotor Virus Mozek Kubis Bunga 35S (CaMV35S) dan dinamakan sebagai pCAMdehD bersaiz 10,592 bp. Pengoptimuman beberapa parameter transformasi berperantaran *Agrobacterium tumefaciens* telah dijalankan termasuklah kepekatan higromisin (40 µg/mL higromisin), dan tahap ketoksikan MCA kepada *N. benthamiana* pada peringkat kultur tisu (60 µg/L MCA) dan pada peringkat pokok (2.0 g/L MCA). pCAMdehD telah dimasukkan ke dalam *N. benthamiana* menggunakan kaedah transformasi berperantaran *Agrobacterium*. Berdasarkan kepada saringan transforman di atas MS media yang mengandungi 60 µg/L MCA, menunjukkan *N. benthamiana* telah berjaya ditransformasikan dengan gen *dehD* dengan 50 % kecekapan transformasi. Integrasi dan pengekspresan *dehD* di dalam *N. benthamiana* telah dibuktikan menggunakan kaedah PCR, Southern Blotting dan transkripsi berbalik PCR. Analisis asai 'leaf-painting' menunjukkan *N. benthamiana* (T₁) transgenik rintang kepada 4.0 g/L MCA berbanding 2.0 g/L bagi pokok kawalan tidak tertransformasi. Analisis Chi Square ke atas kelima-lima pokok transgenik (T₁), mencadangkan gen *dehD* telah tersegregasi mengikut nisbah 3:1 seperti dalam Hukum Mendel. Hasil kajian menunjukkan transgenik *N. benthamiana* yang rintang terhadap herbisid MCA telah berjaya dihasilkan.

TABLE OF CONTENT

CHAPTER	TITLE	PAGE
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGEMENTS	iv
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	xii
	LIST OF FIGURES	xiv
	LIST OF ABBREVIATIONS	xvii
	LIST OF APPENDICES	xviii
1	INTRODUCTION	1
	1.1 Background of the study	1
	1.2 Problem Statement	3
	1.3 Objectives	5
	1.4 Scope of Study	5
2	LITERATURE REVIEW	7
	2.1 Introduction	7
	2.2 Commercial Herbicide and Their Mechanism	8
	2.2.1 Glyphosate	10
	2.2.2 Bromoxynil	13
	2.2.3 Dalapon	15
	2.2.4 Monochloroacetic Acid (MCA)	18
	2.3 Herbicide Resistance Gene	20
	2.4 Bacterial and <i>Rhizobial dehalogenase</i> Gene for Herbicide Resistance	23

2.5	A Proposed <i>dehD</i> Gene and MCA Resistance Transgenic Plant	25
2.6	Plant Transformation System	27
2.6.1	<i>Agrobacterium tumefaciens</i> -Mediated Transformation Method	28
2.6.2	Biolistic	30
2.6.3	Electroporation	31
2.6.4	Polyethylene Glycol (PEG)	32
2.7	T-DNA of Ti Plasmid	33
2.8	Selectable Marker for Plant Transformation	36
2.9	<i>in vitro</i> Regeneration of Transgenic Plant	41
2.10	Transgene integration and expression	44
2.11	Segregation Study According to Mendelian Law	47
2.12	<i>Nicotiana benthamiana</i> as a Model Plant	49
3	METHODOLOGY	52
3.1	Experimental Flow Chart	52
3.2	Plasmid	56
3.2.1	Plasmid pUC57	56
3.2.2	Plasmid pCAMBIA 1305.2	56
3.3	<i>Escherichia coli</i> K12, <i>Escherichia coli</i> DH5- α , <i>Agrobacterium tumefaciens</i> LBA4404	57
3.4	Glycerol Stock Culture	60
3.5	Media for Plant Tissue Culture	60
3.6	Chemicals	61
3.7	Analyses of pUC57dehD	62
3.7.1	Competent Cell <i>E. coli</i> K12 Preparation	62
3.7.2	pUC57dehD Transformation into Competent Cell <i>E. coli</i> K12	62
3.7.3	Plasmid Isolation from <i>E. coli</i>	63
3.7.4	Measurement of DNA Concentration	64
3.7.5	Restriction Enzyme Analyses of pUC57dehD	64
3.7.6	Agarose Gel Electrophoresis	64
3.7.7	DNA Sequencing Analysis	65
3.8	Development of Plant Expression Vector pCAMdehD	65
3.8.1	PCR Amplification of <i>dehD</i> gene	65

3.8.2	PCR Product Purification	66
3.8.3	Restriction Enzyme Digestion	67
3.8.4	Ligation of <i>dehD</i> Gene with pCAMBIA 1305.2	69
3.8.5	Transformation of pCAMdehD into <i>E. coli</i> DH5- α	70
3.8.6	Preparation of Competent Cell <i>Agrobacterium tumefaciens</i> LBA4404	71
3.8.7	Transformation of <i>Agrobacterium tumefaciens</i> with pCAMBIA Vector using Freeze-Thaw Shock Method	72
3.8.8	Plasmid pCAMdehD Isolation and Analyses	72
3.9	Establishment of in vitro <i>N. benthamiana</i> Tissue Culture and Regeneration	73
3.10	Preliminary Test on <i>N. benthamiana</i>	74
3.10.1	Preliminary Test of the MCA Toxicity against Untransformed <i>N. benthamiana</i> at Tissue Culture Stage	74
3.10.2	Preliminary Test of the MCA Toxicity against Untransformed <i>N. benthamiana</i> at Whole Plant Stage	75
3.10.3	Preliminary Test on <i>in vitro</i> Shoot Regeneration and Seed Germination on MS Supplemented with Various Concentration of Hygromycin	76
3.11	Plant Genetic Transformation Studies with New Constructed pCAMdehD	77
3.11.1	Explant Preparation	77
3.11.2	<i>A. tumefaciens</i> Cell Suspension Culture	78
3.11.3	Explant Infection and Co-Cultivation	78
3.11.4	Selection of Transformed Plants	78
3.11.5	Soil Acclimatization	80
3.12	Analyses of Putative T ₀ Transgenic Tobacco Plants	80
3.12.1	Plants Genomic DNA Extraction	80
3.12.2	PCR Analyses	81
3.12.3	Southern Blotting Analysis	83
3.12.4	Total RNA Isolation	86
3.12.5	Quantification of RNA Samples	88

3.12.6	Formaldehyde Agarose (FA) Gel Electrophoresis	88
3.12.7	DNase Treatment and cDNA Synthesis	89
3.12.8	Reverse Transcriptase PCR	90
3.12.9	Comparison Growth Pattern of Transformed and Control <i>N. benthamiana</i> Plants	90
3.12.10	Leaf Painting Assay	91
3.12.11	Mendellian Inheritance Pattern Analysis	91
3.12.12	Chlorophyll Content	92
4	RESULTS AND DISCUSSION	94
4.1	Analysis of pUC57dehD	94
4.1.1	Verification of <i>dehD</i> Gene in the pUC57 Vector	94
4.2	Construction of pCAMdehD	96
4.2.1	PCR Amplification of <i>dehD</i> Gene	96
4.2.2	Digestion of PCR Product and pCAMBIA 1305.2 Vector	99
4.2.3	Ligation of <i>dehD</i> Gene with PCAMBIA Vector	102
4.2.4	Transformation of pCAMdehD into <i>E. coli</i> DH5- α	103
4.2.5	Transformation of Plasmid pCAMdehD into <i>Agrobacterium tumefaciens</i>	105
4.3	Establishment of in vitro <i>N. benthamiana</i> Culture and Regeneration	108
4.4	Preliminary Study	110
4.4.1	Preliminary Test of MCA Toxicity on Non-Transformed <i>N. benthamiana</i> in Tissue Cultures Stage	110
4.4.2	Preliminary Test of the MCA Toxicity against Non-Transformed <i>N. benthamiana</i> at Whole Plant Stage	114

4.4.3	Preliminary Test on in vitro Shoot Regeneration and Seed Germination on MS Supplemented with Various Concentration of Hygromycin.	114
4.5	Plant Genetic Transformation Studies with pCAMdehD	116
4.5.1	<i>Agrobacterium</i> -Mediated Genetic Transformation of <i>N. benthamiana</i>	116
4.5.2	Analysis of Putative Transgenic <i>N.</i> <i>benthamiana</i> Plants	118
4.5.2.1	Polymerase Chain Reaction (PCR) Analyses and DNA Sequencing Analysis	118
4.5.2.2	Southern Blotting Analysis	121
4.5.2.3	Reverse Transcriptase PCR (RT-PCR)	123
4.5.2.4	Comparison Growth Pattern and Phenotype of Transformed and Control <i>N. benthamiana</i> Plants	127
4.5.2.5	Leaf Painting Assay	130
4.5.2.6	Mendelian Inheritance Pattern	131
4.5.2.7	Chlorophyll Content	134
5	CONCLUSION AND FUTURE WORK	139
5.1	Conclusion	139
5.2	Recommendations	141
	REFERENCES	142
	Appendices A-B	174-175

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.1	Properties of monochloroacetic acid (MCA)	18
2.2	Herbicide resistance gene derived from bacteria	23
2.3	Antibiotic and herbicide conditional positive selectable marker genes used in transgenic plant study	39
2.4	Taxonomy of <i>Nicotiana benthamiana</i>	50
3.1	Bacteria used in this study	58
3.2	The composition of plant tissue culture media for <i>N. benthamiana</i>	61
3.3	The <i>dehD</i> gene specific primers (forward and reverse)	65
3.4	PCR amplification scheme	66
3.5	PCR cycling conditions to amplify <i>dehD</i> gene from pUC57dehD to be incorporated into pCAMBIA 1305.2	66
3.6	The dehDF and dehDR primers (forward and reverse)	70
3.7	PCR amplification scheme	71
3.8	PCR cycling conditions to amplify <i>dehD</i> gene from pCAMdehD	71
3.9	Plant growth regulator concentrations in MS medium used for establishment of <i>N. benthamiana</i> tissue culture in vitro	74
3.10	NAD5F and ND5R primers (forward and reverse)	82
3.11	The hptF and hptR primers (forward and reverse)	82
3.12	PCR amplification scheme for <i>NAD5</i> and <i>hpt</i> gene amplification	82
3.13	PCR cycling conditions to amplify <i>hpt</i> and <i>NAD5</i> gene from total genomic DNA plant	83
3.14	DNase I reaction components	89

3.15	Components for cDNA synthesis (first step)	89
3.16	Components for cDNA synthesis (second step)	90
3.17	Samples for chlorophyll content analysis	93
4.1	Effect of different concentration of plant growth regulators in MS medium on shoot regeneration from cultured leaf explants of <i>N. benthamiana</i>	109
4.2	Effect on <i>in vitro</i> shoot regeneration and seed germination on MS supplemented with various concentration of MCA	113
4.3	Preliminary test on MCA toxicity against non-transformed <i>N. benthamiana</i> at whole plant stage	114
4.4	Effect on <i>in vitro</i> shoot regeneration and seed germination on MS supplemented with various concentration of hygromycin	115
4.5	Efficiency of <i>Agrobacterium</i> -mediated transformation of <i>N. benthamiana</i> on selective MS medium	118
4.6	Phenotypic characteristic of transformed and control <i>N. benthamiana</i> plant	129
4.7	Phenotype characteristic of transformed and control <i>N. benthamiana</i> plants based on the number of buds of branches, number of leaflet, start flowering, flower length and weight of the fruit	129
4.8	Percentage of germinated seed of transgenic <i>N. benthamiana</i>	132
4.9	Mendelian Inheritance Analysis of T ₁ progeny, $p(\chi^2_{20.3597}) \leq 3.841$	132
4.10	Chi Square distribution table	133
4.11	Chlorophyll a, b and total chlorophyll content (analysis was done after 7 days of treatment)	135

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
2.1	The reaction catalysed by EPSP synthase (Adapted from Funke <i>et al.</i> , 2006)	11
2.2	Hydrolyzing bromoxynil to its non-toxic benzoic acid by nitrilase enzyme in plants	14
2.3	Chemical structure of dalapon	15
2.4	Degradation process of dalapon (2,2-DCP)	17
2.5	Chemical structure of monochloroacetic acid (MCA)	18
2.6	Proposed hydrolytic mechanism for dehD. An Asp indirectly attacks the carbon bond to the halogen via an activated water molecule. An ester intermediate is not formed.	27
2.7	Overview of the <i>Agrobacterium</i> –plant interaction	35
2.8	Nucleotide sequence of the CaMV 35S promoter	47
2.9	<i>Nicotiana benthamiana</i> plant.	50
3.1	Flow chart of experimental design	52
3.2	Cloning strategy of construction of recombinant plasmid pCAMdehD	54
3.3	The replacement of GUS gene with dehD gene in T-DNA sequence of pCAMBIA	55
3.4	Plasmid pC57dehD construct map	56
3.5	Restriction map of a cloning vector pCAMBIA 1305.2 (Cambia labs, Australia)	57
3.6	Gene ruler 1kb Plus DNA Ladder (Thermo Scientific)	69
3.7	Flow chart of <i>N. benthamiana</i> plant transformation, shoot and root regeneration	79

3.8	Equipment for Southern Blotting	85
4.1	Restriction enzyme analyses on plasmid pUC57dehD	95
4.2	The <i>dehD</i> gene full sequence, restriction enzyme and overhang sequence	97
4.3	PCR products (gradient) of pUC57dehD by using dehDF and dehDR primers	98
4.4	Purification of PCR product (<i>dehD</i> gene)	98
4.5	Digestion product of pCAMBIA 1305.2	100
4.6	Digestion of pCAMBIA 1305.2 and purified PCR products with <i>NcoI</i> and <i>BstEII</i> .	101
4.7	Purified digestion products	101
4.8	Plasmid pCAMdehD	102
4.9	Plasmid extraction products	103
4.10	Restriction enzyme digest at plasmid pCAMdehD and pCAMBIA 1305.2	104
4.11	PCR products on pCAMdehD plasmid recombinant	105
4.12	Products of colony PCR	106
4.13	Digestion products of plasmid recombinant (pCAMdehD from <i>Agrobacterium</i> transformation	106
4.14	Nucleotide BLAST result had shown <i>dehD</i> sequence from pCAMdehD was 100% identical to sequence of <i>Rizobium</i> sp. dehD	107
4.15	Callus formation on <i>N. benthamiana</i> explants (leaf disks) after 2 weeks cultured on MS selective media for preliminary study	111
4.16	<i>N.benthamiana</i> seed germination on MS selective media for preliminary study	112
4.17	Transformed <i>N. benthamiana</i> leaf disks on MS supplemented with 60 µg/L MCA	116
4.18	An overview of <i>Nicotiana benthamiana</i> genetic transformation. (A). <i>N. benthamiana</i> seed	117
4.19	Total genomic DNA <i>N. benthamiana</i>	119
4.20	PCR for NAD5 gene	120

4.21	PCR for <i>hpt</i> gene	120
4.22	PCR for <i>dehD</i> gene	121
4.23	Southern blotting analysis on genomic DNA transformed <i>N. benthamiana</i> digested with <i>NcoI</i>	122
4.24	Total RNA <i>N. benthamiana</i>	123
4.25	PCR for <i>dehD</i> gene on cDNA	124
4.26	Morphological comparison of floral between transformed <i>N. benthamiana</i> and control (non-transformed)	127
4.27	Morphological comparison of fruit between transformed <i>N. benthamiana</i> and control (non-transformed)	128
4.28	Morphological comparison of leaflet between transformed <i>N. benthamiana</i> and control (non-transformed)	128
4.29	Leaf painting assay	130
4.30	Chlorophyll a content for control (wild type) and transgenic <i>N. benthamiana</i> plant after 7 days of MCA treatment	136
4.31	Chlorophyll b content for control (wild type) and transgenic <i>N. benthamiana</i> plant after 7 days of MCA treatment	136
4.32	Total chlorophyll content for control (wild type) and transgenic <i>N. benthamiana</i> plant after 7 days of MCA treatment	137

LIST OF ABBREVIATIONS

2,2-DCP	-	2,2-dichloropropionate
2,4-D	-	2,4-dichlorophenoxyacetic acid
BAP	-	6-benzylaminopurine
BLAST	-	Basic local alignment search tool
C.V	-	Cultivar
CaMV	-	Cauliflower mosaic virus
DehD	-	D-specific dehalogenase
<i>dehD</i>	-	D-specific dehalogenase gene
DIG	-	Digoxigenin
<i>hpt</i>	-	<i>Hygromycin phosphotransferase</i> gene
LB	-	Luria Bertani
MCA	-	Monochloroacetic acid
MS	-	Murashige and Skoog
NAA	-	1-naphthaleneacetic acid
NCBI	-	National Centre for Biotechnology Information
Ri	-	Root inducing
USDA	-	United States Department of Agriculture
Var.	-	Variety
<i>vir</i>	-	Virulence gene

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A	Restriction site of pUC57 plasmid	174
B	Luria Bertani (LB) ingredient and preparation	175

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Weed infestation is a major problem for agricultural activities and these invasive plants directly affect production through competition for nutrients, moisture and light that reduce crop yields to below economic levels, reduce quality of the produce and can render pastures virtually unproductive (Gressel, 2000; Singh and Yadav, 2012). Weed would cost billions in economic losses every year. According to Weed Science Society of America (2016), United State and Canada loses \$43 billion annually in their corn and soybean crops industries. The advent of worldwide industrialization and fast economic development, have boost the cost of farm labor, hence increasing the necessity for cost-effective chemical weed control using herbicides. The increase preference for herbicides for control weed have resulted in worldwide herbicide market which grew by 39% between 2002 and 2011 and it is projected to grow by another 11% by 2016 (Hossain, 2015).

Intensive use of herbicides has been associated with a number of drawbacks such as environmental pollution through surface run-off that leach into deep soil strata and ground water, or adsorption of herbicides in soil (Michaelidou *et al.*, 2000), human and animal health issues (Milosevic and Govedarica, 2002) and most troubling is the evolution of herbicide-resistant weeds (Vencill *et al.*, 2012). As of the beginning of the year 2012, a total of 372 unique, herbicide-resistant weed biotypes have been confirmed in the top 19 countries with intensive agriculture. The United States, Australia and Canada recorded the highest number of herbicide

resistant weeds of 139, 60 and 52 biotypes, respectively (Vencill *et al.*, 2012). Therefore, it is pertinent that alternative methods that would overcome such disadvantages, improve crop yields and productivity would be of significant advantage.

Given the harmful implications of herbicides, development of transgenic crops that are resistant toward specific herbicides using biotechnological method is timely. Herbicides resistance in selected plants involves the addition of a gene coding for an enzyme that detoxifies the herbicide, or encodes for an altered form of an enzyme targeted by the herbicide. In this context, bacterial genes able to degrade toxic compounds are inserted into plants to render new generation of cultivars insensitive to herbicides. Crops displaying resistance to bromoxynil (Taghipour, 2013) and the herbicide Basta and Buster (Zhang *et al.*, 2009) following transformation of a synthetic *bxn* and *bar* gene, respectively, were reported.

Current study will focus using the bacterial genes encodes for production of dehalogenases that cleavage the carbon-halogen bond of the active component of herbicides such as the D-enantiomers monochloropropionate (D-2CP) and monochloroacetate (MCA). The dehalogenase D (DehD) previously isolated from *Rhizobium* sp. RC1 (Berry *et al.*, 1979) that was shown to act specifically on D-2-chloropropionate (D-2CP) and monochloroacetate (MCA) (Huyop and Sudi, 2011).

Broad-spectrum herbicide such as monochloroacetic acid (MCA) is effective at killing a wide range of weeds. Unfortunately, they also kill valuable crops and cause significant losses in agricultural activity. One of the solutions to this problem is by developing herbicide resistant plant for instance using *dehD* gene from Rhizobial system. An application of herbicide resistant plant technology has been reported on many plants and crops such as tobacco (Cicero *et al.*, 2015), rice (Li *et al.*, 2016) and canola (Oliver *et al.*, 2016). In this study we successfully produced plant transformation vector for development of herbicide MCA resistant tobacco cultivar *Nicotiana benthamiana* using the *dehD* gene as herbicide resistance gene and in the same time as a selectable marker gene. The transgenic *N. benthamiana* cultivars

resistant towards the herbicide MCA were obtained and its efficacy in resisting herbicide effects was then evaluated.

From our country perspective, "Plant Biotechnology" has been identified as one of the technologies to accelerate Malaysia's transformation into a highly industrialized country by the year 2020. It has received strong government support and commitment with significant funding for R&D, infrastructure, and human resource development for instance in Kuala Lumpur there was 3rd Plant Genomics Congress on 11-12 April 2016, attended by many researchers all over the country co-hosted by Malaysian Biotechnology Corporation. In Europe, they have started to plan development new crops and cultivars to secure the competitiveness of agriculture. In Strasburg, "The European Plant Science Organisation (EPSO), the European Commission, and the European Association for Bioindustries (EuropaBio) presented the plan entitled "Plants for the Future: A European Vision for Plant Genomics and Biotechnology, for 2025". The document recommends using genetic engineering to achieve some of its goals. Therefore, since Malaysia is still at infant stage since there is no commercialization as yet, at least at fundamental level we will prove that the technology is there to be realized in the near future.

1.2 Problem Statement

The presence of weeds in the farm can adversely affect crop production in a number of ways. Losses may be occurring through the increasing of harvest costs. The greatest cause of economic loss is a reduction in crop yield due to weed rivalry with the crop for available light, nutrients and moisture. Some weeds release toxins that inhibit crop growth, and others may harbor insects, diseases or nematodes that attack crops. Weeds often interfere with harvesting operations, and at times contamination with weed seeds or other plant parts may render a crop unfit for market. Besides that, farmers also spend a lot on weed control activities, labour charges and use of herbicides. Profitable crop production depends on effective weed control.

Many farmers used broad spectrum herbicide such as monochloroacetic acid (MCA) which is cost effective and efficient at killing a wide range of weeds. MCA is a phytotoxic chemical that used as broad spectrum of herbicide against broad leaf weeds, grasses and woody plants (Munn *et al.*, 2005). The high concentration of MCA may kill desired plant that lack of herbicide resistance. Unfortunately, MCA and the others broad-spectrum of herbicide can also kill valuable crops and also causing significant losses in agricultural productivity because the herbicides cannot differentiate between plants that are crops and plants that are weeds.

Efforts should be made to study the production of herbicide-resistant plants that resistant to broad-spectrum herbicides as one of the solution to this problem. Herbicide resistance gene for a wide range of herbicides have been recognised, isolated, characterised and transferred into a wide range of plants leading to rapid progress in the development of herbicide resistance transgenic plants. The *dehalogenase D* gene (*dehD*) encoding dehalogenase enzyme from *Rhizobium* sp. was found to act on monochloroacetic acid (MCA) by cleaves the carbon halogen bond of MCA.

Transferring herbicide resistance genes into agronomically plants is a useful strategy for controlling weeds and increasing agricultural production. However, before transfer the technology into agronomically important plant, the technology must be tested on model plant. In this study, *Nicotiana benthamiana* was used as model plant in this study because this plant can genetically transform and regenerate with good efficiency (Martin *et al.*, 2009).

Therefore, current study is to develop a plasmid containing herbicide resistance gene like *dehD* (previously isolated from *Rhizobium* sp.) and transfer them into a *N. benthamiana* plant via *Agrobacterium tumefaciens*. In addition to the function of *dehD* gene as herbicide resistance gene, it also has potential to be used as selectable marker gene. Transferring herbicide resistance genes such as *dehD* into model plant is a useful strategy for controlling weeds and unwanted plants whereby in the future can be applied for agronomically important plants.

1.3 Objectives

The objectives of this study are as follows:

1. To construct recombinant plasmid pCAMdehD that contains CAMV35S promoter, *dehalogenase D* (*dehD*) gene, NOS terminator and transform the recombinant plasmid into *Agrobacterium tumefaciens*.
2. To transform *Nicotiana benthamiana* plant tissue with *dehD* gene by using *Agrobacterium*-mediated transformation, and the use of *dehD* gene as selectable marker gene.
3. To analyse the integration and expression of the *dehD* gene in transformed *N. benthamiana* plants and verify the T₁ progenies inheritance pattern by segregation analysis.

1.4 Scope of Study

In order to achieve the objectives of this study, six basic research outlined have been proposed:

1. Study the *dehalogenase D* encoding DNA fragment in order to develop a recombinant plasmid construct, pCAMdehD and plant selectable marker based on detoxification of herbicide MCA.
2. Perform preliminary test of the MCA toxicity against untransformed *N. benthamiana* at tissue culture and whole plant level.
3. Transformation of binary plant transformation vector pCAMdehD into *N. benthamiana* plant and the use of *dehD* gene as selectable marker gene in screening stage.
4. Analyses of integration and expression of *dehD* gene into plant genome, by using molecular analyses such as PCR, reverse transcriptase PCR and Southern Blotting analysis.
5. Transgenic plants were analyzed against MCA by leaf painting analysis and chlorophyll content analysis.

6. Segregation analysis using Chi-square analysis was performed to check Mendelian inheritance pattern of *dehD* gene in T₁ generation.

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